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TITLE: Inducing Somatic Pkd1 Mutations in Vivo in a Mouse Model of Autosomal-Dominant Polycystic Kidney Disease

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14. ABSTRACT

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is one of the world's most common life-threatening genetic diseases. Over 95% of diagnosed cases of ADPKD are caused by mutations in *PKD1* or *PKD2* genes. The overall goal of this project is to identify, at the single cell level, the mechanisms that drive the progression of a homozygous *Pkd2* null renal cell towards a pathogenic clonal cyst in a mouse model of ADPKD. We have established two genetic models to induce mutations: one during embryogenesis (Six2-cre) and one in the adult (Villin-cre). The embryonic model has generated clones of wildtype and mutant cells that persist in the adult. The adult model has failed to induce sufficient recombination. In this report we summarize the results obtained with the embryonic model while proposing an alternative approach to increase recombination efficiency.

15. SUBJECT TERMS

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1. **INTRODUCTION:** Autosomal Dominant Polycystic Kidney Disease (ADPKD) is one of the world's most common life-threatening genetic diseases. Over 95% of diagnosed cases of ADPKD are caused by mutations in *PKD1* or *PKD2* genes. The disease is characterized by numerous renal cysts that grow over time, ultimately causing renal failure. A better understanding of the cellular and molecular mechanisms underlying cyst formation could lead to new strategies to prevent the progression of ADPKD. The overall goal of this project is to identify, at the single cell level, the mechanisms that drive the progression of a homozygous *Pkd1* or *Pkd2* null renal cell towards a pathogenic clonal cyst in a mouse model of ADPKD. Ultimately, we aim at identifying the footprint of the pre-cystic cells in order to better target them for directed therapies.
2. **KEYWORDS:** Kidney, renal cystic disease, mouse model, Pkd2, proliferation, primary cilia.
3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?** The following is a list of the major tasks that we proposed would be completed or initiated by the one-year (52 weeks) period of funding.
  - Major Task 1.1: Assess the level of recombination achieved with the Six2–cre and Villin-cre lines using the MADM system (weeks 1-20). 100% completed
  - Major Task 1.2: Histological characterization of the embryonic two-hit model. Do cysts appear? And when? (weeks 1-70). 70% completed
  - Major Task 1.3: To identify defects in Planar Cell Polarity (PCP) associated with Pkd2 somatic mutations and how they correlate with cyst formation (weeks 18-70). 50% completed
  - Major Task 1.4: To identify changes in proliferation associated with Pkd2 somatic mutations and how they correlate with cyst formation (weeks 18-70). 50% completed
  - Major Task 2.1: RNAseq of control vs mutant cells (weeks 36-65). 25% completed
  - Major Task 3.1: Identify the fate of Ddx11-/- cells in our model of ADPKD (weeks 1-50). See section 5 changes/problems.
- **What was accomplished under these goals?**
  1. Major activities:
     a. Mice breeding to generate the desired animal models
     b. Characterization of mutant and wildtype mice
  2. Specific objectives:
     a. Generation of Six2cre; MADM5 tg/gt; Pkd2+/- mice (and control littermates) and analysis of these mice over time

b. Generation of Villincre; MADM5 tg/gt; Pkd2+/- mice (and control littermates) and analysis of these mice over time

c. Generation of Six2cre; MADM5 tg/gt; Pkd2+/-; Ddx11+/- mice (and control littermates) and analysis of these mice over time

3. Significant results and key outcomes:

a. By generating Six2cre; MADM5 tg/gt; Pkd2+/- mice we aimed at inducing homozygous mutations of Pkd2 during embryonic kidney development. Specifically, the Six2cre enhancer drives the expression of cre in the nephron progenitors throughout nephrogenesis. The activity of cre-recombinase will induce rare inter-chromosomal recombination of the MADM5 allele that would result in two sister cells, one eGFP-labeled Pkd2+/+ and the other tdTomato-labeled Pkd2-/-. Our preliminary results confirm the success of this approach. When we analyzed kidneys from these mice at birth we observed the presence of green (wildtype) and red (mutant) clones. We also observed yellow (green and red) clones that are composed of heterozygous cells. See figure 1 for explanation of the model.

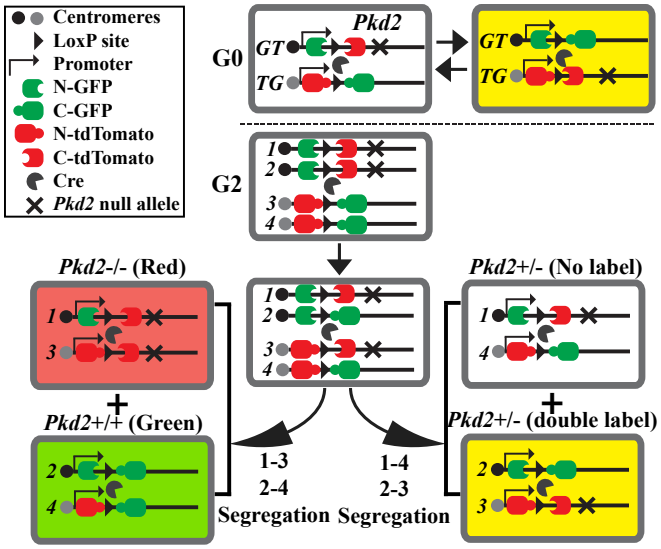
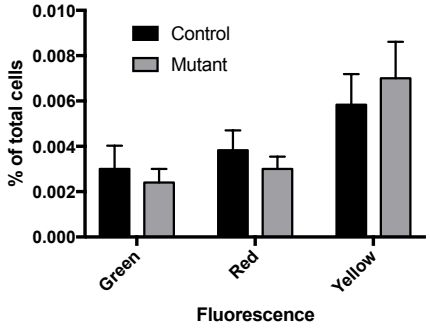


Fig.1. Model to generate kidneys with green(Pkd2+/+), red(Pkd2-/-) and yellow (Pkd2-/+) cells in a non-labeled Pkd2-/+ background. When inter-chromosomal recombination happens at G0 a yellow (Pkd2-/+) cell is generated. When recombination happens at G2 there are two possible outcomes: either a yellow and a non-labeled cell arise (both Pkd2-/+) or a green (Pkd2+/+) and a red (Pkd2-/-) cell arise. In a control mouse that does not have the Pkd2- allele but has cre and MADM5 green, red and yellow cells are generated but they are all wildtype for Pkd2.

While the results obtained were consistent with our expectations, we also observed the following: At birth, mutant clones were morphologically indistinguishable from wildtype clones, with no evidence of cyst development at that stage. Also, the frequency of clones was very low indicating that the cre expression was enough to induce inter-chromosomal recombination but at a relatively low rate.

Fig.2. Kidneys from newborn mutant (Six2cre; MADM5 tg/gt; Pkd2+/-) and control (Six2cre; MADM5 tg/gt; Pkd2+/+) mice were isolated and digested to a single-cell suspension using collagenase (2mg/mL) and dispase (3mg/mL). The suspension was analyzed by flow cytometry and green, red or double-labeled cells were quantified. No significant differences were observed in the percentage of green, red or yellow cells when comparing control and mutant kidneys. The higher frequency of yellow cells is probably attributable to inter-chromosomal recombination at G0.



b. By generating Villincre; MADM5 tg/gt; Pkd2+/- mice we aimed at inducing homozygous mutations of Pkd2 during adult proximal tubule growth. The rationale for this approached was to circumvent a possible severe phenotype with our embryonic kidney model. Unfortunately, the villin enhancer-driven expression of cre was not sufficient to induce recombination of the MADM5 allele at any stage analyzed (newborn, 2 week old and 4 week old mice). Indeed, no fluorescence was observed in those kidneys. However, as shown above, the embryonic induction of recombination did result in fluorescent clones with no severe phenotype so we continued our studies using the Six2cre; MADM5 tg/gt; Pkd2+/- mouse model.

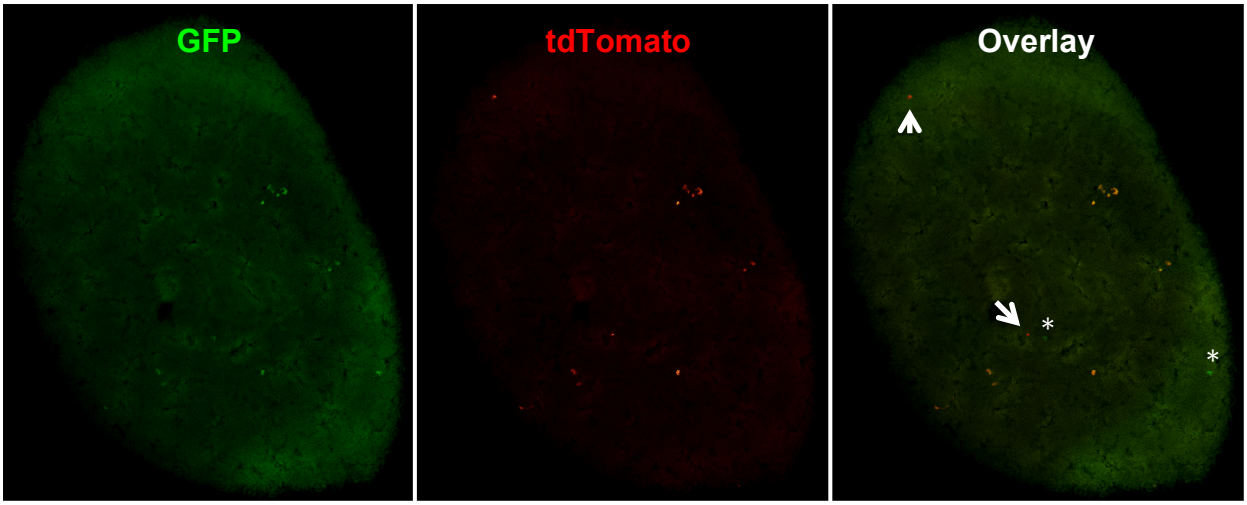


Fig.3. Kidney from 2-week-old mutant (Six2cre; MADM5 tg/gt; Pkd2+/-). Kidneys were isolated, fixed in 4%PFA overnight and processed for vibratome sectioning at 100um thickness. Several clusters of recombined cells are visible. Asterisks point to green-only cells, arrows point to red-only cells.

b. We next aimed at characterizing the timing for cyst generation in the Six2cre; MADM5 tg/gt; Pkd2+/- mice. As mentioned above we did not observe any cyst development from tdTomato positive Pkd2-/- clones in newborn mice. Therefore,

we generated more Six2cre; MADM5 tg/gt; Pkd2+/- mice and allowed them to age. We have currently analyzed mice at 2 weeks (Fig.2 and 3), 4 weeks, 8 weeks and 12 weeks of age. We also have generated a stock of mice to be allowed to age for longer periods of time. Remarkably, while no obvious phenotype is evident at 2, 4 or 8 weeks of age, significant differences in cellular morphology are observed between wildtype (eGFP+) and mutant (tdTomato+) clones at 12 weeks of age. We hypothesize that these differences precede cyst development.

c. The later than expected timing for cyst development has delayed our analysis of proliferation, planar cell polarity, primary cilia and cyst-related markers on the experimental kidneys. However, we have employed this time to optimize the methodological approach to answer those questions.

d. Quantification of green and red cells. If we anticipate no differences in the rate of proliferation nor cell death between wildtype (green) and mutant (red) cells we should observe equivalent numbers of those two cell types in our experimental kidneys as they are generated in a 1:1 ratio. We have analyzed the frequency of green and red cells in newborn mice (fig.2) and observed no significant difference between wildtype and mutant cells.

Stated goals not met:

Of all the stated goals of the project, only one has been compromised: Major Task 3.1: Identify the fate of Ddx11-/- cells in our model of ADPKD (weeks 1-50). The objective of this task was to characterize how ablating mutant cells affected kidney function compared to cyst development. This approach was based on our original proposal to use Pkd1 mutant alleles (in chromosome 17) together with MADM17 (in chromosome 17) and Ddx11 (also in chromosome 17). The inter-chromosomal recombination induced by cre would generate cells that are Pkd1 -/- and also Dds11-/- leading to the death of the cell. As explained in section 5, the MADM17 mouse model got delayed in production while the MADM5 mouse model became available. Pkd2 is in chromosome 5 and, as we stated on the alternative approach section of the proposal, the study of Pkd2 is equally relevant to ADPKD. Hence, we move forward with the goals of the project using MADM5 and Pkd2; however, in this genetic set up the use of Ddx11 became uninformative for the ultimate goal of the project.

- **What opportunities for training and professional development has the project provided?**
  - The project has provided funding to attend the American Society of Nephrology Kidney Week this coming November in Chicago. While the results are not yet ready for

dissemination, we will take advantage of the high quality research on ADPKD presented at the meeting. We anticipate that the discussion with senior leaders in the field will provide an unparalleled opportunity for professional development.

- **How were the results disseminated to communities of interest?**
  - The preliminary nature of the results precludes any comprehensive dissemination at the moment. However, we are active members of the basic nephrology research in University of Michigan and have discussed the details of the projects with basic researchers and clinicians. Once the project is completed with will continue to work with the nephrology community in University of Michigan to disseminate our results.
- **What do you plan to do during the next reporting period to accomplish the goals?**

We are really excited with the preliminary results obtained with the Six2cre; MADM5 tg/gt; Pkd2+/- mice. Our plan for the next reporting period involves:

1. **Further characterization of Six2cre; MADM5 tg/gt; Pkd2+/- mice**.
   a. We will allow Six2cre; MADM5 tg/gt; Pkd2+/- mice and control littermates to age beyond 12 weeks. We currently have mice that are 12 weeks old and will be 28 weeks by the next reporting period. We will be able to analyze the progression of cystic kidney disease in those mice.
   b. Cellular characterization of the Pkd2+/+ and Pkd2-/- kidney cells. We have identified the earliest point (12 weeks) at which mutant and wildtype cells display phenotypical differences. We will use this time point as a reference to identify the emergence of cellular markers and how they relate to cyst development.
2. **Generation of a CMVcre; MADM5 tg/gt; Pkd2+/- mice**. A main set back in our original approach has been the absence of recombination observed when using Villin-cre or very low recombination when using Six2cre. While the amount of recombination is sufficient to study single-cell behavior in Six2cre mice, we were also interested in generating mice with a higher degree or Pkd2 -/- cells recapitulating the more severe phenotype of ADPKD. In order to achieve this goal we will use a CMV-cre transgenic line available from the Jackson Laboratory (strain#006054). In this mouse cre expression is driven by the cytomegalovirus minimal promoter, is expressed from very early embryos and throughout development and adult life. We anticipate that a larger number of recombinant clones will be generated with this mouse.

4. **IMPACT:** Nothing to report

5. **CHANGES/PROBLEMS**

- **Changes in approach and reasons for change:** The objectives and the scope of the proposal have not changed in the reporting period. We have implemented the following experimental changes to our approach:
    - **Exchange of Pkd1 by Pkd2 alleles:**

      The MADM approach to generate single cells with the desired genotype relies on the availability of a MADM construct in the same chromosome where the gene of interest is. Our original approach proposed the use of MADM17 with Pkd1 (in chromosome 17). However, our collaborator Dr. Simon Hippenmeyer experienced a delay in the generation of MADM17. On the other hand, they had just generated MADM5, in chromosome 5 where Pkd2 is located. Pkd1 and Pkd2 function coordinately and disruptions in either gene affect the same pathway results in severe cystic phenotype. Only the frequency of mutations is significantly higher in Pkd1 than Pkd2. As we aim at understanding the developing of cysts once the mutation occurs, we are confident that the exchange of Pkd1 by Pkd2 is not detrimental to the final outcome of the project.
    - **Introduction of CMV-cre:**

      As mentioned above on our future plans for the next reporting period, we will introduce the CMV-cre in our model with the objective of increasing the amount of recombinant cells.
- **Actual or anticipated problems or delays and actions or plans to resolve them**

As mentioned above, the main problem we have encountered is the limited number of recombinant cells we observe per kidney. This has prompted us to re-evaluate the use of stronger cre-reporter lines. We also anticipate that, given the low frequency of recombinant cells in our Six2cre; MADM5 tg/gt; Pkd2+/- mice, the use of fluorescence-activated cell sorting (FACS) to purify those cells may not be ideal as they represent less than 0.01% of the total kidney cells. If sorting these cells using FACS proves unfeasible, we will use laser capture to isolate cells from thick frozen or vibratome sections.

- **Changes that had a significant impact on expenditures** Nothing to report
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents** Nothing to report
- **Significant changes in use or care of human subjects** Nothing to report
- **Significant changes in use or care of vertebrate animals** Nothing to report
- **Significant changes in use of biohazards and/or select agents** Nothing to report

6. **PRODUCTS:**

- **Publications, conference papers, and presentations** Nothing to report
- **Journal publications** Nothing to report
- **Books or other non-periodical, one-time publications** Nothing to report
- **Other publications, conference papers, and presentations.**
    - Presentations: Nephrology division, basic science seminar. University of Michigan. **"New *in vivo* and *in vitro* Models to Study Polycystic Kidney Disease"**. May 5th 2016.
- **Website(s) or other Internet site(s)** Nothing to report
- **Technologies or techniques** Nothing to report
- **Inventions, patent applications, and/or licenses** Nothing to report
- **Other Products** Nothing to report

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

***Example**:*

Name:	Cristina Cebrián-Ligero
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change
Contribution to Project:	No change
Funding Support:	NIH-NIDDK and Pilot grant (see below)

Name:	Yuanyuan Xiao
Project Role:	Laboratory technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change
Contribution to Project:	No change
Funding Support:	No change

Name:	Bryan Torres-Collazo
Project Role:	Undergraduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.25
Contribution to Project:	Bryan performed vibratome sections of wildtype and mutant kidneys
Funding Support:	RISE program

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  The following funding has been awarded to the PI:

  Funding agency: NIH-NIDDK
  Program: (Re)Building a Kidney Partnership Project Program
  Application Title: Transcriptional profiling of in vitro and in vivo derived human kidney tissue using single cell RNA sequencing
  PI: Jason Spence
  Role: Co-I
  Funded period: April 1, 2016 to March 31, 2017
  Amount: $150,000

  Funding source: The Michigan George M. O'Brien Kidney Translational Core Center
  Program: Pilot Grant
  Application tittle: Identification of downstream targets of Etv4 and Etv5 in the murine developing nephron
  PI: Cristina Cebrian-Ligero
  Funded Period: July 15, 2016 to July 14, 2017
  Amount: $40,000

What other organizations were involved as partners? No change

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** *n/a*
   - **QUAD CHARTS:** *n/a*
9. **APPENDICES: No appendices**